THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1993 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 268, No. 6, Issue of February 25, pp. 4161-4169, 1993
Printed in U.S.A.

Identification of Non-cyclooxygenase-derived Prostanoid (F_2-Isoprostane) Metabolites in Human Urine and Plasma*

(Received for publication, October 30, 1992)

Joseph A. Awad, Jason D. Morrow‡, Kihito Takahashi§, and L. Jackson Roberts II¶

From the Departments of Medicine and Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-6602

Free radicals are thought to play an important role in many types of tissue injury. Recently, we reported that a series of prostaglandin F_2-like compounds (F_2-isoprostanes) capable of exerting potent biological activity are produced *in vivo* by free radical-induced lipid peroxidation. Their formation is independent of the cyclooxygenase enzyme and has been shown to increase profoundly in animal models of free radical injury and lipid peroxidation. We now report the identification of F-ring isoprostane metabolites in human urine and plasma utilizing a gas chromatographic/mass spectrometric assay for the major urinary metabolite of prostaglandin D_2 (9α,11β-dihydroxy-15-oxo-2,3,18,19-tetranorprost-5-ene-1,20-dioic acid). Evidence confirming these metabolites as tetranor, dicarboxylic acid compounds containing one double bond, *cis*-cyclopentane ring hydroxyls, and one keto group similar in structure to the major urinary metabolite of prostaglandin D_2 was obtained by analysis of human urine by electron ionization mass spectrometry. Levels of these metabolites in normal human urine were determined and found to be unaffected by cyclooxygenase inhibitors. Evidence that these metabolites arise from F_2-isoprostanes was obtained by demonstrating that (*a*) marked increases in plasma levels and urinary excretion of these metabolites, which were unaffected by coadministration of indomethacin, occurred in rats administered CCl_4 to induce F_2-isoprostane formation and (*b*) marked increases in levels of these metabolites in plasma and urine resulted from the intravenous infusion of F_2-isoprostanes into a rat.

Quantification of these isoprostane metabolites in urine and plasma may provide a reliable index of endogenous isoprostane production which could prove to be an important advance in our ability to assess oxidant stress *in vivo* in humans.

Considerable evidence has implicated reactive free radicals, especially those derived from oxygen, in the pathophysiology of a wide spectrum of disorders including atherosclerosis, ischemia-reperfusion injury, inflammatory diseases, cancer, and aging (1). Peroxidation of lipids is a well recognized sequela of oxidant injury but, despite the variety of methods devised to detect this phenomenon, reliable assessment of lipid peroxidation *in vivo* is still problematic. In fact, this has been a major impediment to investigations in this field (1, 2). Recently, we reported the discovery of a series of prostaglandin (PG)[1] F_2-like compounds in humans that are produced *in vivo* by free radical catalyzed peroxidation of arachidonyl-containing lipids (3). Formation of these compounds is independent of the catalytic activity of the cyclooxygenase enzyme. In conjunction with the Committee on Eicosanoid Nomenclature of the Joint Commission on Biochemical Nomenclature, a facile nomenclature for the individual compounds produced by this mechanism is being developed based on the general term "isoprostane" and the type of prostane ring they contain. In normal human plasma and urine levels of unmetabolized F_2-isoprostanes exceed those of unmetabolized cyclooxygenase-derived prostaglandins by approximately an order of magnitude. In addition, plasma F_2-isoprostane levels have been found to increase up to 500-fold in two animal models of free radical injury and lipid peroxidation, administration of diquat to selenium-deficient rats and CCl_4 to normal rats. These results suggest that quantification of endogenous production of F_2-isoprostanes may provide a valuable tool to assess oxidant stress *in vivo*.

Cyclooxygenase-derived PGs are rapidly metabolized *in vivo*. For this reason, endogenous PG production is best assessed by measurement of stable metabolites in blood or urine. For analagous reasons, quantification of isoprostanes metabolites to assess endogenous production of these compounds might be preferable to quantification of unmetabolized compounds. In addition, there are other advantages associated with F_2-isoprostane metabolite measurement for the assessment of lipid peroxidation. Avoiding the rather facile artifactual *ex vivo* generation of the parent F_2-isoprostanes by autooxidation of arachidonic acid in some biological fluids is one advantage (4). A second advantage arises from the ability of urinary metabolite assays to provide a time-integrated measure of the endogenous production of the compounds of interest. Finally, measurement of unmetabolized cyclooxygenase-derived PGs in urine has been shown to predominantly reflect renal production and, thus, is not a reliable indicator of total endogenous prostanoid production (5, 6). Although, the origin of unmetabolized F_2-isoprostanes in the urine remains to be established, similar constraints may apply to

* This work was supported by National Institutes of Health Grants GM15431, GM42056, ES07028, and HL02499. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Howard Hughes Medical Institute Postdoctoral Physician Fellow.

§ Recipient of a Young Investigator Award from the National Kidney Foundation.

¶ To whom correspondence should be addressed: Dept. of Pharmacology, Vanderbilt University, Nashville, TN 37232-6602. Tel.: 615-322-3304; Fax: 615-322-4707.

[1] The abbreviations and trivial names used are: PG, prostaglandin; PGD-M, 9α,11β-dihydroxy-15-oxo-tetranorprost-5-ene-1,20 dioic acid; TxB_2, thromboxane B_2; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; TLC, thin layer chromatography; EI, electron ionization; MS, mass spectrometry; GC, gas chromatography; NICI, negative ion chemical ionization.

measurement of these compounds in urine as an index of F_2-isoprostane production from extrarenal sources. However, measurement of urinary isoprostane metabolites should provide a reliable index of systemic isoprostane production and lipid peroxidation.

Previous studies examining the metabolic fate of PGD_2 have shown that the major urinary metabolite of this compound in humans is the F-ring PG, 9α,11β-dihydroxy-15-oxo-2,3,18,19 tetranorprost-5-ene-1,20-dioic acid (7). A GC/MS assay for this compound (referred to as PGD-M) was developed utilizing several characteristic features of this molecule to achieve remarkable purification (8). During the development of the PGD-M assay, additional compounds were detected in human urine that appeared chemically similar to the PGD-M molecule but whose production was not affected by cyclooxygenase inhibitors. We now report the identification of these compounds as F_2-isoprostane metabolites in urine and plasma.

MATERIALS AND METHODS

Reagents—Methoxyamine HCl, pentafluorobenzyl bromide, and diisopropylethylamine were obtained from Sigma. Dimethylformamide, undecane and 1-butaneboronic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI). *N,O*-Bis(trimethylsilyl)trifluoroacetamide was obtained from Supelco Inc. (Bellefonte, PA). [$^2H_3$] *N,O*-Bis(trimethylsilyl)trifluoroacetamide and [$^2H_3$]methoxyamine HCl were obtained from Regis Chemical Co. (Morton Grove, IL). All organic reagents were obtained from Baxter Healthcare (Burdick and Jackson Brand, McGaw Park, IL). C-18 Sep-Paks were purchased from Waters Associates (Milford, MA). Thin layer chromatography was performed on silica gel 60ALK6D plates (Whatman International Ltd., Maidstone, Great Britain). Dye mixture No. 1 for silica TLC was obtained from Analtech, Inc. (Newark, DE). Unlabeled PGD-M was chemically synthesized (9). This was then converted to the $^{18}O_4$-labeled derivative for use as an internal standard by the method of Murphy *et al.* (10) involving successive steps of methylation and alkaline hydrolysis with Li^{18}OH.

Prostanoid Analysis—F_2-Isoprostanes were analyzed as described previously (5). Isoprostane metabolites in urine and plasma were isolated and analyzed using a modification of the assay for the major urinary metabolite of PGD_2 (8). For quantitation of F_2-isoprostane metabolites, the major alteration involved TLC purification of *O*-methyloxime, monomethyl ester, mono(pentafluorobenzyl) ester derivatives. The solvent system of acetone/hexane (35:65) was unchanged, and the plates were developed to 13 cm. However the chromatographic standard was changed to Analtech TLC dye No. 1, and the area of silica from which the metabolites were eluted was extended from that described previously for PGD-M. This allowed reproducible inclusion of all the metabolites of interest. The plates were scraped from the leading edge of the yellow band ($R_F = 0.1$) plus 2.3 cm and eluted from the silica with one ml of ethyl acetate.

For EI/MS analysis the procedure was adapted to a larger scale. Briefly, 500-ml aliquots of urine collected from a normal human volunteer during the administration of indomethacin were adjusted to pH 3 with 1 M HCl and allowed to stand for 30 min. This allows PGD-M and similar compounds to undergo endocylization to a hemiketal γ-lactone form. The metabolites were extracted three times from the urine with 0.3 volume of ethyl acetate and the solvent evaporated under reduced pressure. The sample was resuspended in 3 ml of methanol, and 275 ml of H_20 was added and the sample was then sonicated. Ten-ml aliquots were passed over individual C-18 Sep Paks preconditioned with 5 ml of methanol and 5 ml of pH 3 H_20. The columns were then washed with 10 ml of pH 3 H_20 followed by 10 ml of heptane and the metabolites then eluted with 10 ml of ethyl acetate/heptane (50:50). The eluate was combined and evaporated under reduced pressure. The residue was resuspended in 10 ml of methanol, methylated by adding excess ethereal diazomethane for 5 min, after which the solvents were evaporated under N_2 in a 37 °C water bath. The sample was resuspended in 20 ml of acetonitrile, and 80 ml of a 3% aqueous solution of methoxyamine HCl was added and then allowed to react for 30 min at room temperature. Then 400 ml of 50 mM sodium tetraborate buffer, pH 9.1, was added to bring the solution to a pH of 8. Neutral lipids were extracted with ethyl acetate three times (first extraction 500 ml and second and third extraction, 250 ml). The pH of the aqueous solution was adjusted to 2 with 1 M HCl, the metabolites extracted three times from this solution with 250 ml of methylene chloride, and the organic solvent evaporated at reduced pressure. The residue was resuspended in 2 ml of 10% pentafluorobenzyl bromide in acetonitrile and 1 ml of 10% diisopropylethylamine in acetonitrile added. The reaction mixture was placed in a 37 °C water bath, and after 5 min the solvents were evaporated under N_2. The residue was resuspended in 2.5 ml of methanol, sonicated, and applied to TLC lanes in 0.05-ml aliquots. Thin layer chromatography was performed as above. Metabolites were eluted from the silica with ethyl acetate, pooled, and the solvent evaporated under N_2. The trimethylsilyl ether derivatives were formed by adding 40 μl of BSTFA and 20 μl of dimethylformamide and reacting for 30 min at 37 °C.

Formation of the cyclic boronate derivatives and hydrogenation was accomplished as described previously (4, 11). Serum TxB_2 was analyzed following extraction and TLC purification by GC/NICI-MS as described previously (12).

Mass Spectrometry—GC/NICI-MS was carried out as described previously (11). Electron ionization mass spectra were obtained on a Finnigan Incos 50B quadrupole instrument interfaced to a Hewlett-Packard 5890 gas chromatograph. Gas chromatography was performed using a 15 m, 0.25-mm diameter, 0.25-μm film thickness, DB1701 fused silica capillary column (J & W Scientific, Folsom, CA) and an on column injector. The column temperature was programmed from 190 to 300 °C at 2 °C/min. Methane was used as the carrier gas at a flow rate of 1 ml/min. Ion source temperature was 250 °C, electron energy was 70 eV, and filament current 0.25 mA. For analysis, compounds were dissolved in acetonitrile.

Administration of CCl_4 to Rats—Endogenous lipid peroxidation was induced by orogastric administration of CCl_4 (1 ml/kg) to male Sprague-Dawley rats as described (13, 14). Indomethacin or the olive oil vehicle was administered 5 mg/kg subcutaneously at 24, 12, and 1 h prior to the administration of CCl_4 and continued 12 and 23 h after CCl_4 administration. This regimen has been shown previously to inhibit the cyclooxygenase enzyme >90% (15). Urine was collected in 12-h periods from individual animals placed in metabolic cages. Alanine aminotransferase was measured with a spectrophotometric kinetic assay kit from Sigma.

Infusion of F_2-Isoprostanes—Microgram quantities of a mixture of F_2-isoprostanes were isolated from lipid extracts of CCl_4-treated rat livers as follows. Livers from rats 1 h after the administration of 2 ml/kg CCl_4 were harvested and lipids extracted by the method of Folch and Lees as described by Patton and Robbins (16). These lipid extracts contain a mixture of 0.5–1.0 μg of acylated F_2-isoprostanes/g of liver. The lipid extract from 12 g of liver was saponified with 10 ml of MeOH and 10 ml of 15% (w/v) KOH for 30 min at 37 °C. The pH was adjusted to 7–8 with 1 N HCl (22 ml) and free fatty acids extracted with 50 ml of diethyl ether twice. The pH was further lowered to 3, isoprostanes extracted with 50 ml of $MeCl_2$ twice, and the solvent evaporated under reduced pressure. The F_2-isoprostanes were resuspended in 5 ml of methanol and 95 ml of pH 3 H_2O and passed over C-18 and silica Sep-Paks as described for the F_2-isoprostane assay. The F_2-isoprostanes were further purified on a silica TLC system of $CHCl_3$:methanol:acetic acid:H_2O (86:14:1:0.8) developed to 13 cm. $PGF_{2\alpha}$ as a free acid was used as an internal standard (R_F = 0.38) and the plates scraped 1.25 cm on each side of $PGF_{2\alpha}$. A total of 13 μg quantitated as the major F_2-isoprostane peak was isolated from 24 g of liver. The F_2-isoprostane mixture was resuspended in 100 μl of ethanol, 900 μl of phosphate-buffered saline, pH 7.4, and infused into the femoral vein of an anesthetized 250-g Sprague-Dawley rat as described previously (17). Urine was collected in 30-min intervals from catheters placed in the right and left ureters, and blood was sampled via a catheter in the femoral artery.

RESULTS

PGD-M is analyzed by a stable isotope dilution GC/NICI-MS assay as the *O*-methyloxime, monomethyl ester, mono(pentafluorobenzyl)ester, bis(trimethylsilyl) ether derivative (8) (Fig. 1*G*). The major ion in the negative ion chemical ionization mass spectrum of PGD-M is m/z 514 (M − 181, loss of $\cdot CH_2C_6F_5$). The analagous ion generated by the [$^{18}O_4$]PGD-M internal standard is m/z 522. Analysis of human urine for PGD-M reveals two m/z 522 peaks representing the syn- and anti-*O*-methyloxime isomers of [$^{18}O_4$]PGD-M (Fig.

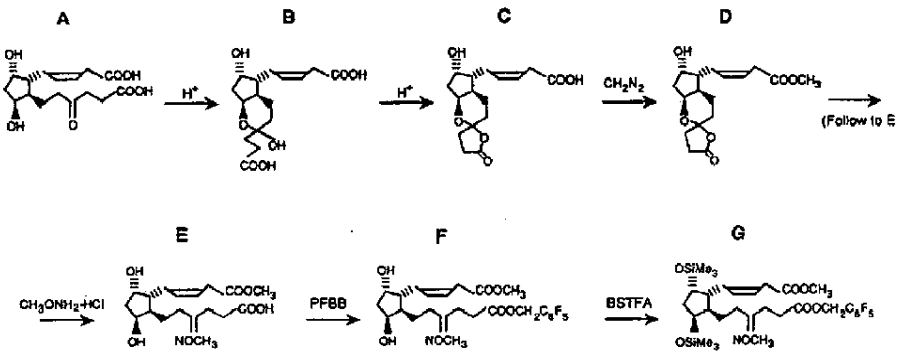

FIG. 1. **Cyclization and derivatization scheme for GC/NICI-MS analysis of the major urinary metabolite of PGD_2 (reproduced by permission, Academic Press).**

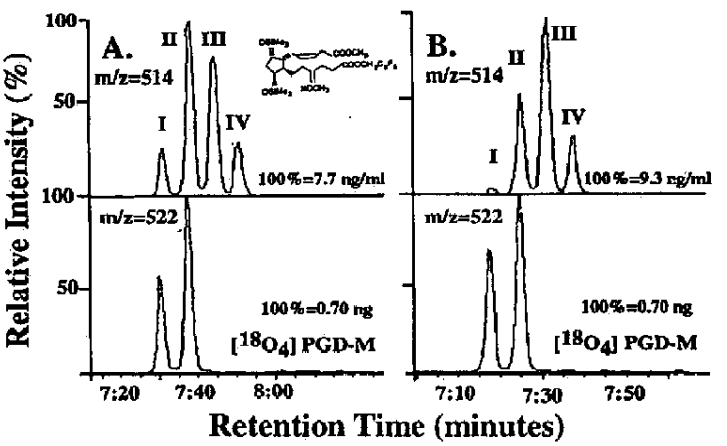

FIG. 2. **Analysis of urine from a normal human volunteer.** *A*, in the absence of indomethacin treatment. *B*, during treatment with indomethacin (200 mg/day).

2*A*). However, in the *m/z* 514 ion current chromatogram, four peaks are present and have been designated by the roman numerals *I–IV*. *Peak I* in the *m/z* 514 ion current chromatogram can be suppressed approximately 90% by cyclooxygenase inhibitors and thus represents an *O*-methyloxime isomer of PGD-M (Fig. 2*B*). On the other hand, *peak II* is only partially suppressed, whereas the remaining peaks are unaffected by cyclooxygenase inhibitors and, therefore, not derived from the activity of that enzyme. In disease states associated with excess PGD_2 production, peaks I and II increase whereas peaks III and IV again remain unchanged. In addition, the ratio of peak I to II representing the *O*-methyloxime isomers of PGD-M becomes identical to that of the internal standard. Since essentially all of the material in peak I is produced by the cyclooxygenase enzyme and thus represents PGD-M exclusively, measurement of this peak has been used to precisely and accurately quantitate endogenous PGD-M (8).

The possibility that the compounds in peaks II, III, and IV which were not suppressed by cyclooxygenase inhibitors might be metabolites of non-cyclooxygenase-derived isoprostanes was investigated. Indirect evidence suggested the possibility that these compounds were structurally similar to the PGD-M molecule. Evidence for this, in part, derives from the constraints imposed by the isolation and derivatization scheme developed for the analysis of PGD-M (Fig. 1). PGD-M is a tetranor dicarboyxlic acid F-ring metabolite of PGD_2. In addition, the C-13 double bond has been reduced and the C-15 hydroxyl group oxidized to a keto group. An important feature of this molecule is the ability of the lower side chain to undergo endocyclization to form a bicyclic hemiketal γ-lactone at acidic pH (Fig. 1*C*). This allows the upper side chain carboxyl group to be selectively methylated along with other carboxylic acid-containing lipids present in the assay mixture (Fig. 1*D*). Subsequent treatment with methoxyamine HCl results in formation of the *O*-methyloxime derivative of the lower side chain keto group, opening of the ring structure, and exposure of the terminal carboxyl group (Fig. 1*E*). At this point, an organic extraction from a neutral buffered solution is performed. This removes the large bulk of acidic lipids that are incapable of cyclizing to protect a carboxyl group from the previous methylation procedure. The PGD-M molecule remains in the aqueous buffer, because the terminal carboxyl group is ionized at neutral pH. The remainder of the assay involves formation of the pentafluorobenzyl ester of the lower chain carboxyl group, a TLC purification step, and formation of the trimethylsilyl ether derivative prior to analysis by GC/NICI-MS.

Given the constraints imposed by the isolation scheme for PGD-M, it seemed probable that the non-cyclooxygenase-derived compounds in Peaks II–IV were structurally similar to PGD-M. Only compounds able to undergo a cyclization that allows protection of a carboxyl group from methylation and then reversal of that cyclization by methoximation to allow formation of a pentafluorobenzyl ester derivative will be detected in the PGD-M assay. In addition, nearly identical TLC and GC characteristics are required for these compounds to be detected in the assay. An identical molecular weight for these compounds was suggested, because all compounds gen-

erate m/z 514 as the predominant ion of their NICI mass spectrum. NICI-MS analysis of fatty acids as a pentafluorobenzyl ester characteristically leads to a single major ion at M − 181 corresponding to the loss of the pentafluorobenzyl moiety.

We then analyzed the compounds as deuterated derivatives to assess whether, like PGD-M, these non-cyclooxygenase derived compounds had two hydroxyl groups and one keto group. Compounds in human urine collected at base line and during the administration of indomethacin, 200 mg, daily were used to form the [$^2H_9$]trimethylsilyl ether derivative of the hydroxyl groups. In both cases, this resulted in a shift of all compounds at m/z 514 to m/z 532 and a shift of the [$^{18}O_4$] PGD-M internal standard from m/z 522 to m/z 540, consistent with the presence of two hydroxyl groups in all compounds. Formation of the [$^2H_3$]*O*-methyloxime derivative caused a 3-Da increase of all compounds consistent with the presence of one keto group. Next, the position and orientation of the hydroxyl groups on these compounds was determined by testing their ability to form a cyclic boronate derivate. Compounds with *cis*-oriented vicinal hydroxyls or hydroxyl groups separated by one carbon can form cyclic boronate derivatives upon incubation with 1-butaneboronic acid. Therefore, only F-ring prostanoids with *cis*-oriented cyclopentane ring hydroxyls will form a cyclic boronate derivative. Because the first step of the metabolism of PGD_2 is its stereospecific reduction to 9α,11β-PGF_2, the PGD-M molecule has *trans*-oriented hydroxyls and cannot form such a derivative (7). However, F_2-isoprostanes have *cis*-oriented hydroxyls on a prostane ring, because they result from the reduction of endoperoxide intermediates in which the peroxide bridge is co-planar (either α,α or β,β). Analysis of human urine with the PGD-M assay, but treated with 1-butane boronic acid prior to formation of the trimethylsilyl ether derivative, showed evidence for both *cis* and *trans* compounds as expected. All of peak I and a portion of the peak II at m/z 514 remain after cyclic boronation. These represent the endogenous PGD-M *O*-methyloxime isomers which cannot form the cyclic boronate derivate. However, the remainder of the compounds in peaks II–IV shifted from m/z 514 to m/z 436, which corresponds to the M − 181 ion of the cyclic boronate derivative. Similar treatment of human urine collected during the administration of indomethacin to suppress PGD-M production suggested that only *cis* hydroxyl compounds remained with shift of all compounds from m/z 514 to m/z 436 (data not shown).

The presence and number of double bonds were then determined by hydrogenation of the compounds prior to GC/MS analysis and observing for a shift in mass of the major ion. After hydrogenation, the compounds were analyzed by monitoring the m/z 514, 516, 518, and 520 ion current chromatograms. Hydrogenation resulted in the disappearance of essentially all of the compounds at m/z 514 as new peaks appeared at m/z 516 only, consistent with the presence of one double bond. These peaks had retention times approximately 40 s longer than those of the original compounds. However, a set of peaks remained in the m/z 516 ion current chromatogram at the same retention time as the unhydrogenated compounds, despite the disappearance of the compounds at m/z 514. Therefore, the presence of the peaks at m/z 516 at the same retention time as the m/z 514 peaks I and II prior to hydrogenation could not be accounted for by an isotope effect of lower mass compounds. Analysis of urine from an individual treated with indomethacin to suppress PGD-M production illustrates this fact (Fig. 3*B*). Monitoring m/z 514, 516, and 522 revealed that the pattern of the peaks in the m/z 516 ion

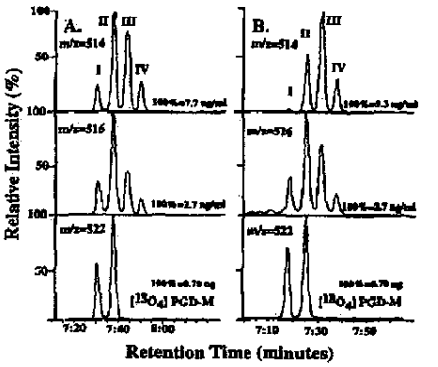

FIG. 3. **Analysis of urine from a normal volunteer including monitoring of m/z 516.** *A*, in the absence of indomethacin treatment. *B*, during treatment with indomethacin (200 mg/day).

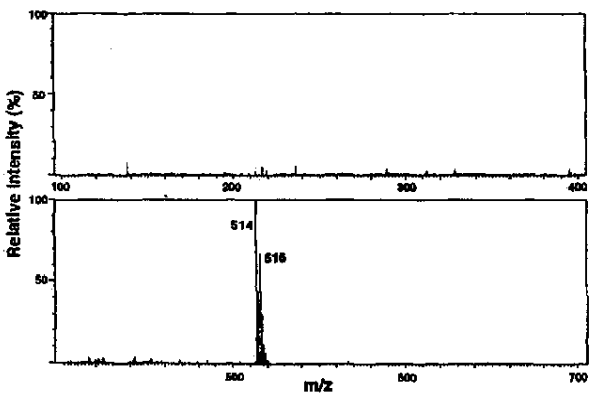

FIG. 4. **NICI full scan mass spectrum of Peak II of urine from a human volunteer during treatment with indomethacin (200 mg/day).**

current chromatogram is different than that of the m/z 514 ion current chromatogram. If the peaks in the m/z 516 ion current chromatogram were merely the result of the isotope effect of the m/z 514 compounds, the pattern should have been identical. Instead, whereas peak I is very small relative to the other peaks at m/z 514 in urine from an indomethacin-treated individual, this ratio is increased at m/z 516. In addition, whereas peak III is the dominant peak in the m/z 514 ion current chromatogram, peak II is the dominant peak at m/z 516. This is further illustrated by examining the full NICI mass spectrum of peak II in urine taken from an individual on indomethacin (Fig. 4). This spectrum reveals a doublet at m/z 514 and m/z 516, which verifies the presence of compounds with a predominant ion of m/z 516. The full NICI mass spectrum of peak I was similar, whereas with peaks III and IV the intensity of of m/z 516 relative to m/z 514 was no greater than that expected from the distribution of naturally occurring isotopes (data not shown). These data suggested the presence of additional compounds at m/z 516 which lack a double bond and elute from the GC at the same retention time as peaks I and II. Using deuterated derivatives as outlined above it was confirmed that these compounds also have two hydroxyl groups and one keto group like the m/z 514 compounds. In addition, these compounds formed a cyclic boronate derivative resulting in a shift of the M − 181 ion to m/z 438 consistent with the presence of *cis*-oriented hydroxyl groups on a prostane ring (data not shown).

The chemical evidence above was consistent with the hy-

pothesis that the additional compounds seen in the PGD-M assay at m/z 514 and m/z 516 were F-isoprostane metabolites of similar structure to PGD-M. To obtain structural confirmation, purification of these compounds from an indomethacin-treated human volunteer in quantities sufficient for electron ionization mass spectrometry was undertaken as detailed under "Materials and Methods." A representative EI mass spectrum of the urinary metabolites is presented in Fig. 5*B* and compared with the EI mass spectrum of chemically synthesized PGD-M analyzed as the *O*-methyloxime, monomethyl ester, mono(pentafluorobenzyl) ester, bis(trimethylsilyl) ether derivative (Fig. 5*A*). The mass spectra of the metabolites revealed the same molecular ion as that of PGD-M at m/z 695. In addition, other anticipated high molecular weight ions are also present and are identical to those of PGD-M. In this and other scans (not shown), a doublet of 2 mass units higher was seen for most higher molecular weight ions including the molecular ion. This is consistent with the existence of metabolites lacking a double bond as suggested from the analysis by NICI-MS described above. Consistent with this interpretation, a doublet was not observed for the m/z 492 ion (M-113–90). In the mass spectrum of PGD-M this ion results

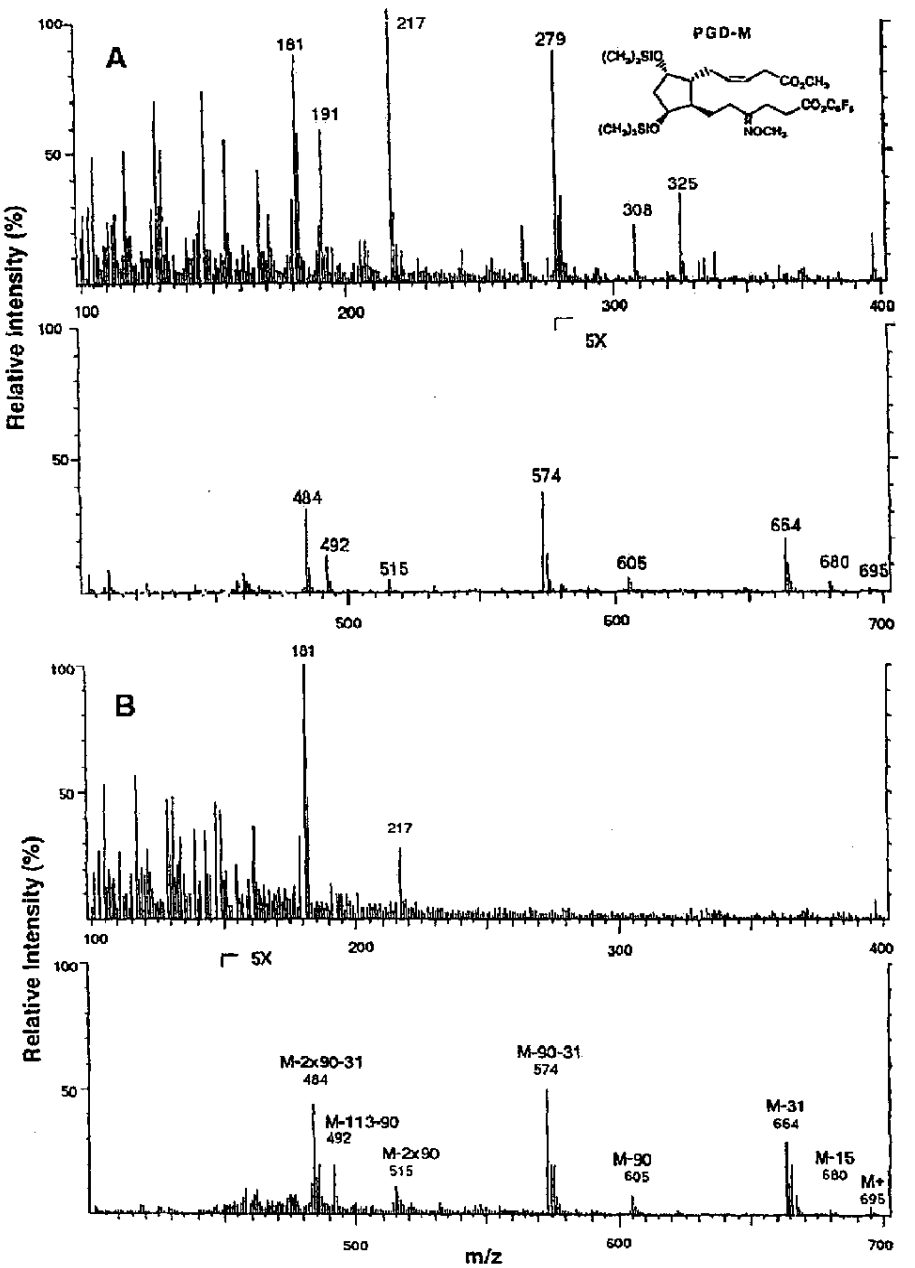

FIG. 5. **EI mass spectrum of chemically synthesized PGD-M (*A*) and of urine metabolites from a human volunteer during indomethacin treatment (200 mg/day) (*B*).** *Inset* in *A* shows structure of PGD-M molecule analyzed.

from the loss of 113 Da ($\cdot CH_2CH{=}CHCH_2COOCH_3$) plus 90 Da ($Me_3SiOH$) from the molecular ion. Therefore, both metabolites with a double bond and metabolites in which the double bond has been reduced would generate the *m/z* 492 ion. The ions noted in the mass spectrum of PGD-M at *m/z* 279, 308, and 325 were not observed in the mass spectra of the non-cyclooxygenase-derived metabolites. These ions result from loss of various combinations of a portion of the lower side chain and a trimethylsilyl group from the parent molecule. Given that this mass spectrum represents a mixture of the probable structures for the isoprostane metabolites proposed below (see "Discussion" and Fig. 7), and that the side chain configuration that yields these ions would be present in only some of the metabolites, it might be expected that the intensity of these ions in the mass spectra of isoprostane metabolites may not be identical to that of PGD-M.

We then determined the feasability of quantitating these isoprostane metabolites in human urine. Only measurement of peak III and IV at *m/z* 514 seemed reasonable as an index of the metabolites (Fig. 3). Peak II at *m/z* 514 is a mixture of PGD-M and isoprostane metabolites. Similarly, peaks I and II at *m/z* 516 represent both isoprostane metabolites whose major M − 181 ion is *m/z* 516 and contributions from peaks I and II at *m/z* 514 by an isotope effect. Therefore, quantitation of peaks III and IV at *m/z* 514 was done by comparing the ratio of their peak heights with the average of the peak heights of both *O*-methyloxime isomer derivatives of the [$^{18}O_4$] PGD-M internal standard at *m/z* 522. This contrasts with the method used to calculate endogenous PGD-M in which only the ratio of the peak heights of the isomer in peak I are used for quantitation. This method is necessary for PGD-M, because peak II is contaminated by isoprostane metabolites, and it is valid because PGD-M is measured in comparison with its own stable isotope internal standard which undergoes derivatization in an identical fashion to the native molecule. Potential problems arise when measuring a compound by comparison with a similar but nonidentical stable isotope internal standard, as is the case with the isoprostane metabolites in peaks III and IV. To deal with the assay to assay variation in the ratio of *O*-methyloxime isomers in peaks I to II of the internal standard, the peak heights were averaged to represent the quantity of internal standard added. Using this method of quantitation, the precision for the measurement of peaks III and IV at *m/z* 514 was determined by analysis of five 1-ml aliquots of urine from a human volunteer. The precision was ± 11% and ± 12% for peaks III and IV, respectively. This compares with a precision of ± 4.0% determined for PGD-M (peak I at *m/z* 514) in the same experiment when calculated as described above. Next, we determined the normal range in 16 healthy adult volunteers (7 men and 9 women) taking no medications. This yielded values of 4.2 ± 2.7 and 1.7 ± 1.0 ng/mg creatinine for peaks III and IV, respectively. There was no significant difference in the values obtained for males and females. In view of previous reports that urinary metabolites of some eicosanoids can also be detected in the circulation, we assessed whether the isoprostane metabolites could also be detected in plasma. Plasma (2–4 ml) from 10 healthy volunteers (5 males and 5 females) was assayed by the same method described for urine except that the sample pH was adjusted to 3 prior to adding the [$^{18}O_4$]PGD-M internal standard. This is done to prevent exchange of ^{18}O with ^{16}O by plasma esterases (10). Peaks III and IV were quantified as described above and yielded values of 15 ± 4.9 and 7.1 ± 4.3 pg/ml for peaks III and IV, respectively.

We observed previously that pharmacological doses of indomethacin and a number of other cyclooxygenase inhibitors (ibuprofen, naproxen, and tolmetin) did not qualitatively suppress production of the isoprostane metabolites (8). Having developed a reliable method for determining their levels, we sought to confirm this observation quantitatively. Two male volunteers collected 24-h urine specimens on at least three occasions in a base-line state and during administration of 50 mg of indomethacin orally four times/day. Levels of peak I representing endogenous PGD-M production only and peak III representing the isoprostane metabolites were quantified as described above (Table I). The levels of PGD-M (Peak 1) were suppressed ≥88% consistent with marked inhibition of the cyclooxygenase enzymes, whereas, peak III metabolite levels were not decreased by indomethacin treatment.

Following administration of CCl_4 to rats to induce endogenous lipid peroxidation, circulating levels of unmetabolized F_2-isoprostanes increase dramatically (3). In order to obtain further evidence that the urinary metabolites we identified were metabolites of F_2-isoprostanes, we assessed whether these metabolites also increased in this model. When normal rat urine was analyzed the major urinary metabolite of PGD_2 (PGD-M) was not detected. However, the *m/z* 514 and *m/z* 516 peaks representing the putative F_2-isoprostane metabolites were present with levels from approximately 0.2 to 2.0 ng/mg creatinine. Rat urine collected for 12 h after the administration of 1 ml/kg CCl_4 showed 2–35-fold increases in levels of these compounds compared with urine collected 12 h prior to CCl_4 administration (Table II). Furthermore, these increases were not suppressed by the administration of indomethacin. Serum TxB_2 was measured at the end of the experiment 24 h after CCl_4 administration to confirm the adequacy of cyclooxygenase inhibition. TxB_2 levels were 80 ± 61 ng/ml (n = 7) in rats administered the olive oil vehicle and 0.61 ± 0.26 ng/ml (n = 6) in rats treated with indomethacin, an inhibition of >99%. The extent of liver damage in the vehicle

TABLE I

Effect of indomethacin on PGD-M and non-cyclooxygenase-derived prostanoid metabolite levels

PGD-M (peak I, *m/z* 514) and non-cyclooxygenase-derived prostanoid metabolite (peak III, *m/z* 514) levels in two volunteers in the absence of and during treatment with indomethacin (200 mg/day).

	Peak I, *m/z* 514	Peak III, *m/z* 514
	ng/mg creatinine	
Subject 1		
Base line	1.00 ± 0.00	2.70 ± 0.30
Indomethacin	0.12 ± 0.04	3.80 ± 0.80
Subject 2		
Base line	1.10 ± 0.30	3.50 ± 1.20
Indomethacin	0.12 ± 0.20	3.30 ± 0.80

TABLE II

Effect of CCl_4 administration on rat urinary F_2-isoprostane metabolite levels

F_2-isoprostane metabolite levels in urine collected from rats 12 h before and after administration of 1 ml/kg CCl_4 orogastrically. One group of animals was treated with indomethacin per protocol and the other the olive oil vehicle (six to eight animals used per group).

Conditions	F_2-isoprostane metabolite			
	Peak II, *m/z* 514	Peak III, *m/z* 514	Peak I, *m/z* 516	Peak II, *m/z* 516
	ng/mg Cr			
Pre-CCl_4				
Vehicle	0.2 ± 0.1	0.8 ± 0.4	0.2 ± 0.1	0.6 ± 0.2
Indomethacin	0.3 ± 0.1	2.1 ± 0.6	0.4 ± 0.1	1.0 ± 0.4
Post-CCl_4				
Vehicle	1.7 ± 0.8	4.1 ± 1.6	7.0 ± 2.8	11.2 ± 5.0
Indomethacin	2.2 ± 0.4	4.3 ± 1.1	6.1 ± 2.3	10.1 ± 4.0

controls and indomethacin-treated animals after CCl_4 was not significantly different as assessed by plasma alanine aminotransferase determinations at 24 h (controls 1275 ± 789 IU/liter *versus* indomethacin 1159 ± 645 IU/liter). In a similar experiment we determined whether these metabolites could be detected in plasma and whether their levels were increased after the administration of CCl_4. In this experiment four animals were sacrificed 4 h after the administration of 1 ml/kg CCl_4, and four control animals were sacrificed after the administration of the corn oil vehicle. Plasma levels of the isoprostane metabolites were determined to be 8–20-fold increased after the administration of CCl_4 (Table III). Verification that the urinary compounds which increased after the administration of CCl_4 to rats were identical to the non-cyclooxygenase-derived metabolites identified in human urine was obtained from two sources. Analysis using deuterated derivatives confirmed the presence of one keto group and two hydroxyl groups, whereas formation of cyclic boronate derivatives allowed assignment of the hydroxyl group orientation as *cis*. Furthermore, coinjection experiments of urine samples taken from an indomethacin-treated individual and a CCl_4-treated rat revealed perfect cochromatography of the peaks of interest at *m/z* 514 and *m/z* 516 by GC/MS.

We then sought to obtain direct confirmatory evidence that these metabolites identified are metabolites of F_2-isoprostanes by determining whether intravenous infusion of purified F_2-isoprostanes into a rat results in increased levels of the metabolites in the urine and plasma. Prior to infusing the F_2-isoprostanes, base-line urinary metabolite excretion for the rat was determined from a 20-h collection in a metabolic cage. Then the animal was prepared for the infusion as described under "Materials and Methods." After a 30-min equilibration period, a 30-min infusion of F_2-isoprostanes (13 μg) was initiated and urine collected for 3 h in 30-min fractions. Blood obtained at the end of the infusion was found to have 1650 pg/ml plasma of the major unmetabolized F_2-isoprostane peak, whereas 150 min after the infusion was terminated, F_2-isoprostane levels had returned to normal (15 pg/ml). Urine from hours 2 and 3 after the initiation of the infusion was pooled, assayed for isoprostane metabolites, and the excretion rate compared with that measured during the base-line period. The post-infusion excretion rate was found to be 470–850% above base line (Fig. 6*A*). Metabolite levels were also determined in plasma 150 min after the F_2-isoprostane infusion was terminated and found to be 400–1200% above levels in a control animals infused with the 10% ethanol vehicle (Fig. 6*B*). Control infusion of 10% ethanol also showed no increase in urinary metabolite excretion. These findings provide direct evidence that the metabolites identified arise from metabolism of F_2-isoprostanes.

DISCUSSION

Oxidative stress has been suggested to play a role in a wide variety of disease processes (1). Unfortunately, the importance of oxidative stress and resultant lipid peroxidation in these processes is difficult to assess in humans due to a lack of reliable methods to assess oxidant stress *in vivo*. Despite the variety of methods developed for the quantification of lipid peroxidation, most of these suffer from well recognized limitations when used *in vivo* to assess oxidant status (1, 2). Two important limitations are those of sensitivity and specificity. F_2-isoprostanes can be easily detected in normal human biological fluids and their production increases markedly in established animal models of oxidant injury. Thus, quantification of these products of lipid peroxidation may represent an important advance in our ability to assess oxidant stress *in vivo*.

TABLE III

Increase in F_2-isoprostane metabolites in plasma after administration of CCl_4

Plasma F_2-isoprostane metabolite levels from control rats and 4 h after the administration of CCl_4 (n = 4 per group).

Conditions	Plasma F_2-isoprostane metabolite		
	Peak III, *m/z* 514	Peak I, *m/z* 516	Peak II, *m/z* 516
		pg/ml	
Control	3 ± 3	7 ± 8	9 ± 9
CCl_4	60 ± 18	71 ± 21	71 ± 20

We now report the identification of urinary metabolites of F_2-isoprostanes that were detected in the course of developing a mass spectrometric assay for the major urinary metabolite of cyclooxygenase-derived PGD_2 (PGD-M). This assay can also be used for the quantification of these metabolites in urine and plasma. The major compounds identified are 16 carbon compounds which share the same molecular weight and chemical functionalities of the PGD-M molecule, *i.e.* two hydroxyl groups, a keto group, and one double bond. Lesser amounts of a metabolite(s) that does not contain any double bonds was also detected. All compounds were shown to have *cis*-cyclopentane ring hydroxyl groups as would be expected of F_2-isoprostane metabolites. The mechanism F_2-isoprostane formation results in the production of four regioisomers of this compound (4). As discussed under "Results," only prostanoid metabolites capable of forming a hemiketal lactone bicyclic structure will be detected by in the PGD-M assay. Knowledge of the structure of the four parent F_2-isoprostane regioisomers, the functional groups determined to be present on the F-isoprostane metabolites, and the constraints imposed by the isolation procedure allows one to propose possible structures for the principal metabolites that could be detected by this assay (Fig. 7). Metabolites other than the ones depicted may be present but are less likely to be formed. For example, rather than the double bond adjacent to the keto group being reduced, it is possible that the double bond on the other side chain has been reduced. However, the double bond adjacent to the keto group of prostaglandin metabolites is usually preferentially reduced compared with the other double bond (7, 18–20). Isomers of each of the basic structures shown in Fig. 7 may also be present because of the difference in stereochemistry of the side chains and the cyclopentane ring hydroxyls. The prostane ring hydroxyls must be *cis* because they derive from reduction of endoperoxide intermediates but they can be oriented either α,α or β,β. In addition, the side chains of isoprostanes are predominantly, although not exclusively, oriented *cis*, resulting in side chains that can be either α,α or β,β with respect to the prostane ring (21–23). Determination of the stereochemistry of these metabolites is not possible at this time as isolation of sufficient quantities of the metabolites in pure form from urine for NMR studies is not feasible. Given the differences in stereochemistry, it is of considerable interest that the F_2-isoprostanes can, in fact, be metabolized via the routes described for cyclooxygenase-derived prostanoids. The presence of predominantly *cis* side chains and nonstandard side chain configurations might be predicted to alter the metabolism of the parent F_2-isoprostane compounds.

The identification of these tetranor F_2-isoprostane metabolites may help to explain findings published by Nugteren in 1975 (24). Using a novel mass spectrometric assay for the simultaneous determination of tetranor metabolites of all prostaglandins irrespective of prostane-ring subtype from

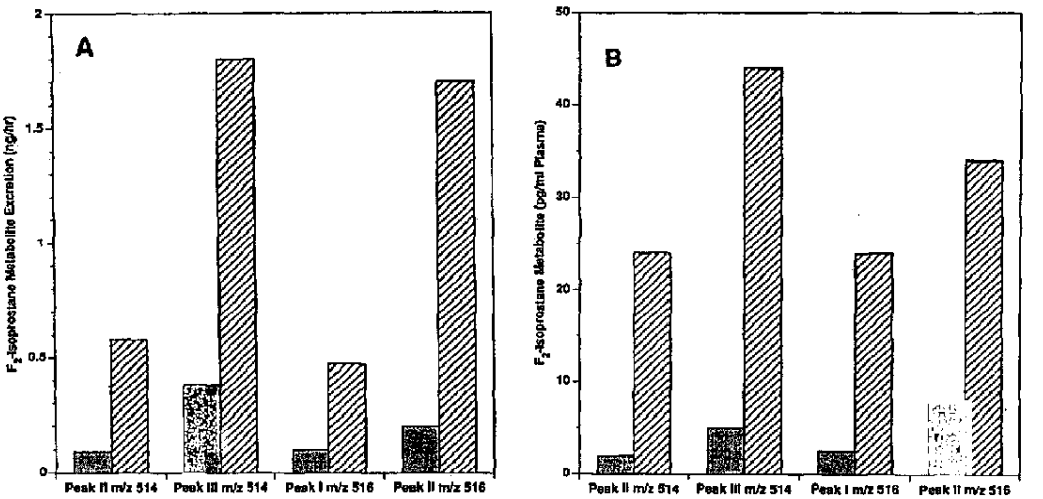

FIG. 6. *A*, increase in urinary F-isoprostane metabolite excretion after infusion of F_2-isoprostanes into a rat. *B*, increase in plasma F-isoprostane metabolite levels 150 min after infusion of F_2-isoprostanes *versus* those measured after infusion of vehicle into a control rat. *Solid bars*, control; *hatched bars*, post F_2-isoprostane infusion.

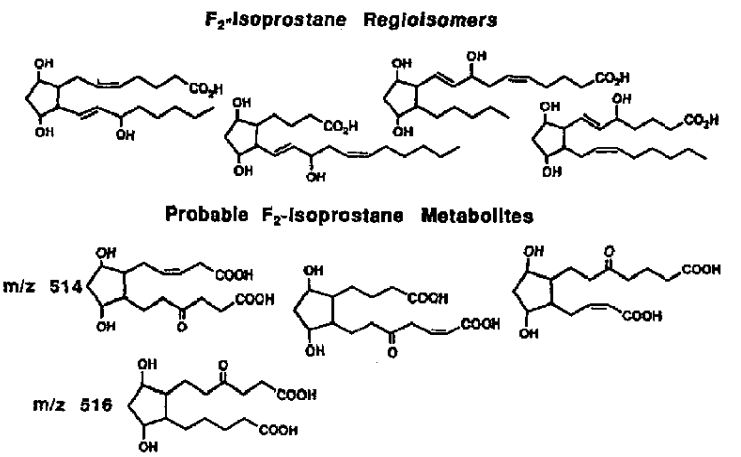

FIG. 7. **Structures of unmetabolized F_2-isoprostane regioisomers (*top panel*) and the metabolites most likely to be detected in the assay at *m/z* 514 and *m/z* 516 (*bottom panel*).**

which they were derived, Nugteren found an approximately 10-fold higher level of total endogenous prostaglandin in humans than could be accounted for by previous studies of prostaglandin metabolism. In retrospect, this discrepancy may now be explained, in part, by the likely possibility that Nugteren's assay also detected tetranor F_2-isoprostane metabolites.

The ability to quantify metabolites of F_2-isoprostanes potentially offers distinct advantages compared with quantification of the unmetabolized compounds. First, this circumvents the problem of artifactual generation of unmetabolized compounds by autooxidation *ex vivo*. We previously demonstrated that F_2-isoprostanes are readily formed *in vitro* by autooxidation of arachidonate-containing biological fluids such as plasma (4). However, this potential artifact can be totally eliminated by measuring metabolites of these compounds. Second, the primary source of the unmetabolized F_2-isoprostanes in human urine has not yet been determined. In particular, it is not known whether urinary F_2-isoprostanes predominantly reflect local production of these compounds in the kidney or filtration of the compounds from plasma and, therefore, systemic production. In the case of unmetabolized cyclooxygenase-derived PGs in urine, it has been shown that these compounds are produced almost entirely in the kidney (5, 6). Thus, until analagous information has been obtained with respect to unmetabolized F_2-isoprostanes in urine, quantification of these compounds in urine may not be a reliable or sensitive indicator of isoprostane formation from extrarenal sites. However, quantification of F_2-isoprostane urinary metabolites should provide a reliable means to assess total endogenous formation of isoprostanes. A third advantage of measuring urinary isoprostane metabolites is that they provide a time-integrated estimate of isoprostane production. Quantification of unmetabolized F_2-isoprostanes in a biological sample, such as blood, only provides an instantaneous measure of isoprostane production. This process might vary considerably with time and errors in estimating the overall magnitude of the production of these compounds may be introduced by the selection of sampling times. The delayed appearance of the F_2-isoprostane metabolites in urine and plasma may be advantageous. In the F_2-isoprostane infusion experiment circulating levels of the unmetabolized F_2-isoprostanes had returned to normal by 150 min after the infusion was terminated. However, F_2-isoprostane metabolite lev-

els in plasma and urine at that time were severalfold above normal. Therefore, in situations where biological fluids cannot be obtained for analysis at the time an acute but limited pathophysiological event occurs, isoprostane release can still be assessed by measuring isoprostane metabolites in biological fluids collected at a time distant to the event.

In summary, we have identified urinary metabolites of F_2-isoprostanes that can be quantified using a stable isotope dilution mass spectrometric assay initially developed for quantification of the major urinary metabolite of PGD_2. These metabolites can also be detected at lower concentrations in plasma. Quantification of F_2-isoprostane metabolites may provide a new and valuable tool for the assessment of oxidant injury *in vivo* in humans.

REFERENCES

1. Halliwell, B., and Grootveld, M.(1987) *FEBS Lett.* **213**, 9–13
2. Slater, T. F. (1984) *Methods Enzymol.* **105**, 283–293
3. Morrow, J. D., Hill, K. E., Burk, R. F., Nammour, T. M., Badr, K. F., and Roberts, L. J., II (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 9383–9387
4. Morrow, J. D., Harris, T. M., and Roberts, L. J., II (1990) *Anal. Biochem.* **184**, 1–10
5. Frölich, J. C., Wilson, W., Sweetman, B. J., Smigel, M., Nies, A. S., Carr, K., Watson, J. T., and Oates, J. A. (1975) *J. Clin. Invest.* **55**, 763–770
6. Catella, F., Nowak, J., and Fitzgerald, G. F. (1985) *Am. J. Med.* **81**, Suppl. 2B, 23–29
7. Liston, T. E., and Roberts, L. J., II (1985) *J. Biol. Chem.* **260**, 13172–13180
8. Morrow, J. D., Prakash, C., Awad, J. A., Duckworth, T. A., Zackert, W. E., Blair, I. A., Oates, J. A., and Roberts, L. J., II (1991) *Anal. Biochem.* **193**, 142–148
9. Prakash, C., Saleh, S., Roberts, L. J., Blair, I. A., and Taber, D. F. (1988) *J. Chem Soc. Perkin Trans. I*, 2821–2826
10. Murphy, R. C., and Clay, K. L. (1982) *Methods of Enzymol.* **86**, 547–551
11. Pace-Asciak, C., and Wolfe, L. S. (1971) *J. Chromatogr.* **56**, 129–133
12. Dworski, R., Sheller, J. R., Wickersham, N. E., Oates, J. A., Brigham, K. L., Roberts, L. J., II, and Fitzgerald, G. A. (1989) *Am. Rev. Respir. Dis.* **139**, 46–51
13. Burk, R. F., Lawrence, R. A., and Lane, J. M. (1980) *J. Clin. Invest.* **65**, 1024–1031
14. Burk, R. F., and Lane, J. M. (1979) *Toxicol. Appl. Pharmacol.* **50**, 467–478
15. Jackson, E. K. (1989) *Adv. Prostaglandin Thromboxane Leukotriene Res.* **18**, 322–326
16. Patton, G. M., and Robbins, S. J. (1990) *Methods Enzymol.* **187**, 195–215
17. Badr, K. F., DeBoer, D. K., Schwartzberg, M., and Serhan, C. N. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3438–3442
18. Granstrom, E., and Samuelson, B. (1971) *J. Biol. Chem.* **246**, 7470–7485
19. Hamberg, M., and Samuelson, B. (1971) *J. Biol. Chem.* **246**, 6713–6721
20. Roberts, L. J., II, Sweetman, B. J., and Oates, J. A. (1981) *J. Biol. Chem.* **256**, 8384–8393
21. Pryor, W. A., and Stanley, J. P. A. (1975) *J. Org. Chem.* **40**, 3614–3615
22. Pryor, W. A., Stanley, J. P. A., and Blair, E. (1976) *Lipids* **11**, 370–379
23. O'Conner, D. E., Mihelich, E. D., and Coleman, M. C. (1984) *J. Am. Chem. Soc.* **106**, 3577–3584
24. Nugteren, D. H. (1975) *J. Biol. Chem.* **250**, 2808–2812